Application of Retinol to Human Skin *In Vivo* Induces Epidermal Hyperplasia and Cellular Retinoid Binding Proteins Characteristic of Retinoic Acid but Without Measurable Retinoic Acid Levels or Irritation

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We investigated the clinical, histologic, and molecular responses of normal human skin to all-*trans*-retinol (ROL) application, compared to those induced by topical all-*trans*-retinoic acid (RA), and measured ROL-derived metabolites. Up to 1.6% ROL, 0.025% RA in vehicle (70% ethanol/30% propylene glycol), or vehicle alone were applied in a double-blind fashion to normal buttock skin and occluded for 4 d. ROL produced from none to only trace erythema, which was clinically and statistically insignificant, whereas RA induced a significant 3.7-fold increase in erythema score compared to vehicle ($n = 10$, $p < 0.01$). However, ROL induced significant epidermal thickening (1.5-fold at 1.6% ROL, $p < 0.01$), similar to RA (1.6-fold at 0.025% RA, $p < 0.01$), relative to the vehicle. ROL, compared with vehicle, also increased mRNA levels of cellular retinoic acid binding protein (CRABP-II) and cellular retinol binding protein (CRBP) genes as determined by Northern analysis (5–6-fold and 6–7-fold, respectively) and riboprobe *in situ* hybridization. CRABP-II and CRBP protein levels were also higher following ROL than vehicle treatment, as measured by ligand binding (3.2-fold, $p < 0.001$; $n = 7$) and Western analysis (3.6-fold, $p < 0.003$; $n = 6$), respectively. Epidermal retinyl ester (RE) content, measured after removal of stratum corneum, rose 240-fold ($p < 0.005$, $n = 5$) by 24 h of ROL occlusion. RA content, however, was undetectable or detectable only at trace amounts in all samples obtained at 0, 6, 24, and 96 h after ROL occlusion. Detectability of RA was not correlated with ROL treatment (compared to untreated normal skin, $p = 0.86$) or baseline skin ROL levels (average $r = -0.1$, $p > 0.3$). These data demonstrate that ROL application 1) produces trace erythema not significantly different from vehicle, whereas RA causes erythema; 2) induces epidermal thickening and enhances expression of CRABP-II and CRBP mRNAs and proteins as does RA; 3) causes marked accumulation of retinyl ester; and 4) does not significantly increase RA levels. Taken together, the data are compatible with the idea that ROL may be a prohormone of RA, because it produces changes in skin similar to those produced by RA but without measurable RA or irritation. *J Invest Dermatol 105:549–556, 1995*

All-*trans*-retinol (ROL), better known as vitamin A_1, assumes a critical role in vision, spermatogenesis, and cellular growth and differentiation [1]. As the predominant retinoid in circulation, ROL is delivered to cells bound to retinol binding protein. Within cells, it is converted to many different retinoids depending upon cell need and type. In human skin, besides ROL, retinyl esters (RE), 3,4-didehydroretinyl esters, and all-*trans*-retinoic acid (RA) are present [2]. Although ROL is believed to be a precursor of other retinoids, the metabolic pathways operative in skin physiology and pharmacology are not well understood. Notwithstanding, ROL is widely used in cosmetics and toiletries as a GRAS (generally recognized as safe) ingredient in the U.S. The concentration of ROL in these products is usually between 0.1% and 1.0%, but can be as high as 5% [3].

A drawback of topical RA in clinical use [4–6] has been local skin irritation manifested as mild erythema and stratum corneum peeling. Therefore, a retinoid with activity similar to RA but without local irritation would be of interest. In cells RA is thought to be formed enzymatically from ROL by a two-step oxidation process in which retinal is the intermediate metabolite [7]. *In vitro* work with human keratinocytes [8] and *in vivo* studies with mouse skin [9] indicate the presence of a similar two-step process. In

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Abbreviations: EtOH/PG, ethanol/propylene glycol; RA, all-*trans*-retinoic acid; RE, retinyl esters; ROL, all-*trans*-retinol.

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addition, the presence of a similar enzymatic system in human skin was detected in psoriatic epidermis and at very low levels in normal skin [10]. For these reasons, we asked whether in normal human skin *in vivo*, RA-mediated effects may be achieved by ROL application.

Recently, an occlusive patch test was developed for assessing topical retinoid action in human skin *in vivo*. Four days of continuous RA occlusion reproducibly induces erythema clinically with epidermal hyperplasia and spongiosis [11]. The clinical and corresponding histologic responses mimic skin changes seen after chronic (4 months or longer) unoccluded daily topical RA treatment [5]. These clinical and histologic findings are not RA specific because a skin irritant such as 2% sodium lauryl sulfate can induce similar changes in the 4-d patch test. However, only RA, and not sodium lauryl sulfate, will enhance the expression of cellular retinoic acid binding protein (CRABP)-II mRNA [12]. We have used this patch-test system to compare the activity of ROL with RA and to study retinoid metabolism in normal human skin *in vivo* after application of ROL. Here we report that ROL treatment of human skin 1) is much less erythemogenic than RA; 2) is similar to RA in its ability to induce epidermal hyperplasia, and to increase the expression of CRABP-II and cellular retinol binding protein (CRBP) mRNAs, and proteins; 3) is actively metabolized and stored as RE; and 4) does not result in significant RA accumulation.

MATERIALS AND METHODS

Materials Isocitrate, isocitrate dehydrogenase, 13-*cis*-RA, all-*trans*-RA, and all-*trans*-ROL were purchased from Sigma Chemical Co., St. Louis, MO. [^{3}H] all-*trans*-RA was obtained from DuPont NEN, Boston, MA. 9-*cis*-RA was a gift of Drs. Joseph Grippo, Arthur Levin, P.F. Sorter, and A.A. Liebman of Hoffmann La-Roche, Nutley, NJ. RA metabolites 4-hydroxy-RA, 4-*oxo*-RA, 13-*cis*-4-*oxo*-RA, and 5,6-epoxy-RA were gifts from Michael Rosenberger and P.F. Sorter of Hoffmann-La Roche, Nutley, NJ. Retinyl esters were gifts from Dr. Anders Vahlquist, University of Linköping, Sweden and Dr. Christine Huselton of Hoffmann-La Roche, Nutley, NJ. High-pressure liquid chromatography (HPLC) grade solvents were used for extractions and chromatography. The Sperisorb ODS-1 column was obtained from Phase Separations, Norwalk, CT. Horseradish peroxidase labeled anti-rabbit IgG antibody was purchased from Cappel, Cochranville, PA. Enhanced chemiluminescence detection reagent was obtained from Amersham, Arlington Heights, IL.

Human Subjects and Biopsy Procedures Adult normal volunteers had varying concentrations of ROL (up to 1.6%), 0.025% RA, and vehicle (EtOH-PG) applied to buttock skin (100 μl/18 cm^2) according to a computer-generated randomization code. The sites were then occluded under plastic wrap secured with surgical tape, and covered with light-proof dressing. After 4 d, the occlusive dressing was removed and the degree of erythema present at each site was scored according to a 10-point scale (0, none; 1–3, mild; 4–6, moderate; 7–9, severe) [11]. Keratomed sections of skin (2 cm × 6 cm wide, and 0.2 mm in thickness) and 4-mm punch biopsies were obtained under a 1% lidocaine anesthesia from each site [13]. Each punch biopsy was bisected. Half was placed in 10% neutral-buffered formalin and processed for hematoxylin and eosin histology and the other half was embedded in Optimal Cutting Temperature medium (OCT, Miles Laboratories, Elkhart, IN), frozen in liquid nitrogen and stored at −70°C until used for *in situ* hybridization analyses. Keratome biopsies were immediately snap-frozen in liquid nitrogen and stored at −70°C until used for RNA isolation, ligand binding, and Western and HPLC analysis. In six subjects, keratome biopsies were performed after ROL occlusive patches were in place for 0, 6, 24, and 96 h. Stratum corneum (the most superficial, nonviable, keratinized layer of skin) was removed by tape stripping of each site used for the time course prior to biopsy. Adequacy of stratum corneum removal was confirmed by frozen section histology of a 2-mm punch biopsy obtained from each tape-stripped patch site. All subjects gave written consent in a protocol approved by the University of Michigan Institutional Review Board.

Histology Punch biopsies processed to hematoxylin and eosin were read at the light microscope level by one investigator (SWK) who was blinded as to which treatment group each specimen represented. Stratum corneum compaction and epidermal spongiosis were assessed using a 0 to 4 ordinal scale where 0 was none and 4 was maximum. The number of granular cell layers was counted and epidermal thickness in micrometers was measured from the bottom of the stratum corneum to the epidermal basement membrane at five inter-rete ridge sites and the measurements averaged.

RNA Isolation and Northern Blot Analysis of Treated Skin Total RNA was isolated from keratome biopsies obtained as described above. The guanidinium isothionate–cesium chloride procedure was used as previously described [14], except that cesium trifluoroacetate (Pharmacia) was used according to the manufacturer's instructions. RNA samples (40 μg total RNA, determined by OD_{260}) were electrophoretically size-fractionated on 1% formaldehyde-agarose gels and transferred to derivatized nylon membrane (Zeta-Probe, Bio-Rad). Blots were sequentially hybridized against ^{32}P-labeled CRABP-II [15], CRBP (a gift of Dr. William Blaner), and 36B4 [16]. DNA probes were prepared by random priming, and quantitated using a phosphorImager (Molecular Dynamics) as previously described [15]. Hybridization to the CRABP-II and CRBP probes were normalized as previously described [14,15], except that 36B4 rather than cyclophilin was used as the control probe. Results have been expressed as fold change relative to the ethanol-propylene glycol vehicle applied to the same subject.

***In Situ* Hybridization** *In situ* hybridization techniques were similar to those previously described [17,18], with slight modification. Briefly, cryostat sections (5 μm thick) were thaw-mounted onto slides coated with 3-aminopropyl (triethoxy) silane and fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline. After subjected to proteinase K digestion, sections were post-fixed with 4% paraformaldehyde and acetylated with 0.25% (v/v) acetic anhydride in 0.1 M triethanolamine, pH 8.0. Prehybridization was performed in a buffer containing 50% formamide, 0.6 M NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM ethylenediaminetetraacetic acid, 50 μg/ml Heparin, 10 mM dithiothreitol, 10% polyethylene glycol, and 1 × Denhardt's solution. Hybridization was carried out in fresh prehybridization buffer supplemented with 0.5 mg/ml denatured salmon sperm DNA, 0.5 mg/ml yeast tRNA, and 1 μg/ml digoxigenin-labeled cRNA probe for 20 h at 50°C. Following hybridization, the sections were washed in 2 × SSC (1 × SSC = 8.77 g/l NaCl; 4.41 g/l Na citrate, pH = 7.0), then digested with RNase A for 30 min at 37°C to remove non-hybridized excess cRNA probe. Hybridization signals were detected by immunohistochemistry using a commercially available kit (Nucleic acid detection kit, Boehringer Mannheim Co, Indianapolis, IN). To ascertain the probe specificity and background hybridization, digoxigenin-labeled sense cRNA for CRABP-II and CRBP were hybridized to the sections as control.

Quantitation of CRABP Protein Keratome biopsies under liquid nitrogen were ground to a fine powder by mortar and pestle. The powdered tissue was then homogenized in 20 mM Tris, pH 7.4, containing 10 mM thioglycerol, 10% glycerol, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride using a glass homogenizer. Soluble material was obtained by centrifugation at 100,000 × *g* for 60 min at 4°C and used as the source for CRABP. For measurement of CRABP I and CRABP II, high-speed supernatants (100 μg protein) were incubated with 50 nM [^{3}H] all-*trans*-RA (37.1 Ci/mmol) in homogenization buffer, containing 0.006% Ammonyx LO, for 16 h in the dark. CRABP I and CRABP II were resolved and quantified by fast protein anion-exchange liquid chromatography as described by Sanquer and Gilchrest [19]. CRABP II and CRABP I were eluted with retention times of 5 to 6 and 12 to 14 min, respectively. Quantitation of CRABP I and CRABP II was verified using purified recombinant CRABP I and CRABP II proteins [20]. Protein content in the supernatants was measured by the PIERCE/BCA (Pierce Co, Rockford, IL) microplate method using bovine serum albumin as standard.

Western Blotting of CRBP Protein Soluble proteins (100 μg) were separated on 15% sodium dodecylsulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was then treated with 5% TBST (5% nonfat dried milk in 20 mM Tris, pH 7.6, containing 137 mM NaCl, 1% Tween 20) blocking solution for 2 to 4 h. After a wash with TBST, the membrane was incubated for 16 hours with affinity purified anti-rat CRBP I polyclonal antibody (a gift from Dr. William Blaner). Following another wash with TBST, the membrane was then incubated with horseradish peroxidase labeled anti-rabbit IgG secondary antibody for 2 to 4 hours at 1:5000 dilution. After a final wash with TBST, the membrane was reacted with enhanced chemiluminescence detection reagent for 1 min and immediately exposed to autoradiographic film to visualize the protein bands.

Extraction and Separation of Retinoids and Retinyl Esters Extraction procedures were the same as given previously [21]. Retinoids were separated by reverse-phase HPLC using a Hewlett-Packard 1090M ChemWorkstation with a diode array detector and a Spherisorb ODS-1 column as previously described [21]. When extracted material was also evaluated for retinyl ester content, the procedure was modified as follows: the flow rate was changed to 1 ml/min of solvent B (acetonitrile:0.2 M ammonium

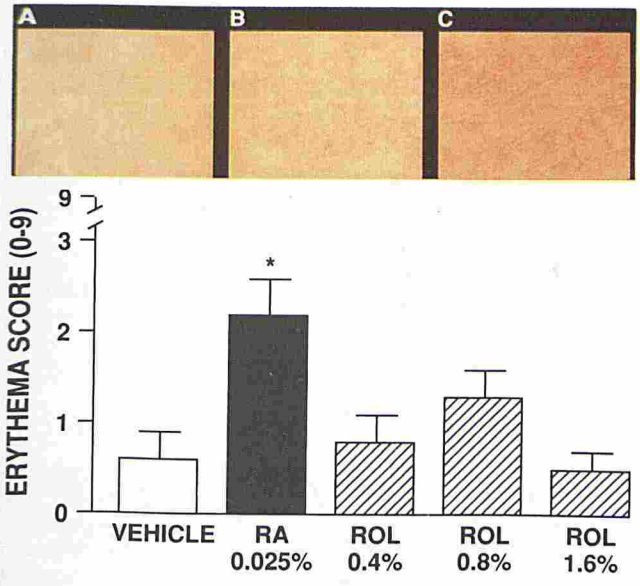

Figure 1. RA but not ROL treatment of human skin *in vivo* produces significant erythema in a 4-d patch test. Clinical photograph of a representative volunteer's skin treated for 4 d under occlusion with vehicle (*A*); 1.6% ROL (*B*); and 0.025% RA (*C*). The human skin bioassay conditions and procedures are detailed in *Materials and Methods*. *D*) Erythema score of vehicle, RA-, and ROL-treated sites. Data represent mean ± SEM, n = 10. *p < 0.01 versus vehicle.

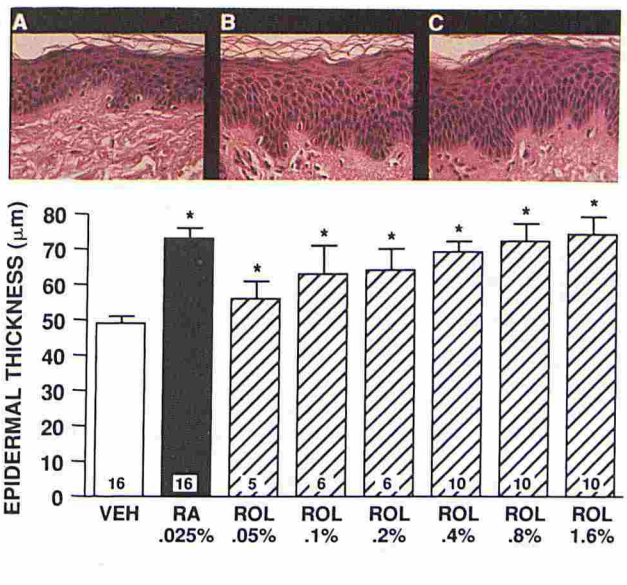

Figure 2. Histology corresponding to the clinical photograph of Fig 1*A–C*: *A*) vehicle; *B*) 1.6% ROL; *C*) 0.025% RA. *D*) Epidermal thickness of skin treated for 4 d under occlusion with various concentrations of ROL in EtOH/PG vehicle, vehicle (VEH) alone, or 0.025% RA. Values at the bottom of each *bar* represent number of subjects studied (n). *p < 0.05 versus vehicle. Measurement of epidermal thickness is described in *Materials and Methods*.

acetate:acetic acid of 19:1:0.008) at 40 min and the time for separation was extended to 115 min. The eluant from the HPLC flowed directly into a scintillation spectrometer (Radiomatic-A295) to determine recovery of retinoids.

Statistical Methods Comparisons of mean levels among treated sites of various clinical and histologic parameters were performed with either the paired t test or the repeated measures analysis of variance and the Tukey procedure for multiple group comparisons. All p values are two sided. Summary statistics are expressed as means ± SEM. Data analysis was performed with the use of the Michigan interactive data analysis system (MIDAS), a statistical software package developed at the Center for Statistical Consultation and Research at the University of Michigan.

RESULTS

Clinical Response of Normal Human Skin to ROL and RA Under Occlusion for 4 d The clinical response observed at patch sites from a representative volunteer is shown in **Fig 1*A–C***. ROL, up to 1.6%, did not induce significantly more erythema than vehicle in the 4-d bioassay. Only RA produced erythema that was clinically evident and statistically significant compared with vehicle (3.7-fold increase in erythema score, $p < 0.001$; n = 10) **(Fig 1*D*)**.

Histologic Changes Induced by ROL and RA Application to Human Skin Although clinically inactive following 4 d of occlusion, when examined histologically ROL (1.6%) significantly increased epidermal thickness (74 μm ± 5) as compared with vehicle (50 μm ± 3) ($p < 0.01$; n = 10). The magnitude of increased epidermal thickness induced by ROL (1.6%) was similar to 0.025% RA (79 μm ± 4) **(Fig 2*A–C*)**. Increase in epidermal thickness by ROL was dose dependent. ROL-induced epidermal thickening began to plateau at 0.4% with half-maximal response occurring between 0.05% and 0.1% **(Fig 2*D*)**. Like RA, ROL increased the number of mitotic figures in the epidermis and enhanced epidermal spongiosis. (For complete histologic parameters, see **Table I**; n = 10.)

Induction of CRABP-II Gene Expression by ROL in Human Skin ROL application significantly induced CRABP-II mRNA expression in keratomed tissue relative to vehicle. The results of Northern blot analysis from a representative volunteer's specimen are shown and data for the subjects studied are summarized in **Fig 3**. The maximum level of CRABP-II mRNA induction (6.2-fold over vehicle) by ROL was reached at 0.2% and was comparable to that induced by 0.025% RA. Riboprobe *in situ* hybridization similarly demonstrated a marked increase of CRABP-II transcripts in ROL-treated skin compared with vehicle **(Fig 4)**. Expression of CRABP-II mRNA, although faint, was evident in vehicle-treated skin. In epidermis, vehicle-treated skin demonstrated a patchy staining pattern, whereas in ROL-treated skin the signal was confluent and dense. In the upper reticular and entire papillary dermis and around blood vessels, cells with dendritic and spindle-shaped morphology showed increased staining intensity in ROL-treated skin compared with vehicle. The *in situ* hybridization staining signal was comparable between RA and ROL-treated skin (data not shown). Pretreatment of the tissue with RNase A abolished the positive staining observed with CRABP-II anti-sense riboprobe. Furthermore, the *in situ* hybridization signal was not observed when sense probe was utilized, demonstrating the specificity of the CRABP-II riboprobe.

Induction of CRBP Gene Expression by ROL in Human Skin The results of Northern blot analysis of specimens from four volunteers are shown in **Fig 5**. Relative to vehicle, ROL treatment significantly increased (6.3-fold, $p < 0.01$; n = 4) expression of CRBP mRNA in keratomed skin. By riboprobe *in situ* hybridization, increase in CRBP gene transcripts induced by ROL was visualized throughout the epidermis as well as the upper reticular and entire papillary dermis within dendritic and spindle-shaped cells **(Fig 4)**.

Measurement of CRABP and CRBP Protein Levels in ROL, RA, and Vehicle-Treated Human Skin Protein levels of CRABP-I and CRABP-II in vehicle-treated normal skin were 0.37 ± 0.26 and 2.86 ± 0.40 pmol/mg protein (n = 7), respectively. In comparison with vehicle, ROL (1.6%) application increased the level of CRABP-II protein to 8.95 ± 2.22 pmol/mg (3.2-fold, $p < 0.001$; n = 7) as determined by ligand binding to the two CRABP proteins resolved by anion-exchange chromatography. The

Table I. Comparison of Epidermal Histology Following 4-d Patch Test[a,b]

Histologic Parameter	Vehicle (EtOH/PG) (n = 10)	0.025% RA (n = 10)	0.4% ROL (n = 10)	0.8% ROL (n = 10)	1.6% ROL (n = 10)
Stratum corneum compaction	1.1 ± 0.2[c]	1.7 ± 0.3[c]	1.5 ± 0.3[c]	1.3 ± 0.3[c]	1.5 ± 0.3[c]
Granular layer thickness	1.0 ± 0.1[c]	1.8 ± 0.2[d,e]	1.4 ± 0.1[c,d,e]	1.5 ± 0.1[d]	1.7 ± 0.1[d]
Spongiosis	0.4 ± 0.1[c]	1.7 ± 0.2[d]	1.4 ± 0.1[d]	1.5 ± 0.2[d]	1.7 ± 0.1[d]
Mitosis/3 hpf	0.5 ± 0.2[c]	1.8 ± 0.2[d]	1.4 ± 0.3[d]	1.5 ± 0.3[d]	1.2 ± 0.1[c,d]
Epidermal thickness (μm)	50 ± 3[c]	79 ± 4[d]	69 ± 3[d]	72 ± 5[d]	74 ± 5[d]

[a] All values are mean ± SEM.
[b] All parameters are scored on a 0 to 4 scale, except for epidermal thickness and mitotic figures.
[c,d,e] For each parameter, pairwise comparison of means with at least one superscript in common denotes non-significant difference at the 0.05 level. For example, granular layer thickness for the vehicle-treated site is significantly less than the site treated with 0.025% RA but not significantly different from the site treated with 0.4% ROL because of the common superscript *c*.

CRABP-II protein elevation to 8.62 ± 1.92 pmol/mg produced by 0.025% RA (3.1-fold, $p < 0.001$; n = 6) was comparable to that induced by 1.6% ROL treatment **(Fig 6*A*)**. CRABP-I constituted 10% (range, 0–22%; n = 7) of the total CRABP protein in vehicle-treated normal skin (detection limit of 0.019 pmol). Although compared with vehicle, ROL and RA treatment reduced CRABP-I protein levels by 13% (to 0.30 ± 0.09 pmol/mg, n = 7) and 11% (to 0.31 ± 0.09 pmol/mg, n = 7), respectively, these reductions were not statistically significant. Relative to vehicle, ROL (1.6%) treatment significantly increased the CRBP protein level by 2.8-fold ($p < 0.003$; n = 6) as measured by Western analysis **(Fig 6*B*)**. Similarly, RA (0.025%) treatment raised CRBP protein levels (2.9-fold, $p < 0.005$).

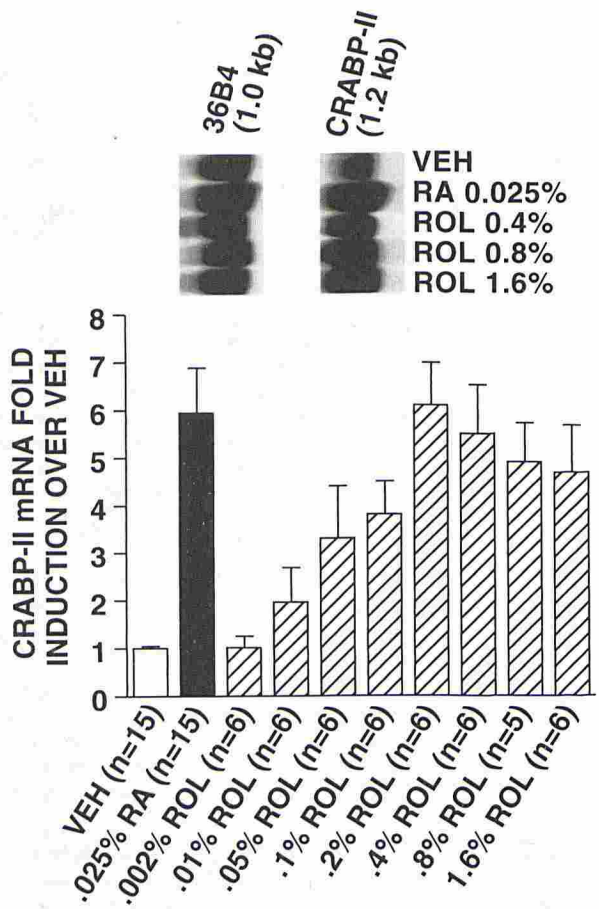

Figure 3. Northern analysis of CRABP-II gene expression following 4-d occlusive ROL treatment. *Top*, results shown are from a representative subject. *Bottom*, dose response of ROL treatment. Normalized CRABP-II mRNA levels were determined as described in *Materials and Methods*.

Retinoid Profile in Human Epidermis Following ROL Treatment Four days of continuous ROL 0.4% occlusion increased skin content of ROL, 13-*cis*-ROL, and RE in comparison with vehicle **(Fig 7)**. A detectable increase in RA levels, however, was not observed (detection limit, 1 ng). These values are a mean of five of the six subjects studied to better reflect the physiologic skin retinoid profile at time 0. One participant, who was excluded from the analysis, had basal ROL, 13-*cis*-ROL, and RE levels of 1561, 242, and 894 ng/g wet weight, respectively. These values were at least fourfold greater than the highest level observed for each retinoid amongst the other five subjects. Upon questioning, this participant revealed that he primarily consumes vegetables (including carrots). Even in this subject with the highest baseline ROL levels, RA was not detected before or following ROL treatment. Measured over time (0, 6, 24, and 96 h), quantities of ROL, 13-*cis*-ROL, and RE in stratum corneum-free, ROL-treated skin increased significantly by 24 h (ROL, 70-fold, $p < 0.02$; 13-*cis*-ROL, 280-fold, $p < 0.03$; RE, 260-fold, $p < 0.005$; n = 5) **(Fig 8)**. At each time-point studied, RA was undetectable or found at trace levels only in all samples (total of 20). The frequency with which RA was detected from ROL-treated specimens (five of 15) was similar to that seen in untreated normal skin (one of five), and RA levels between the time points were comparable ($p = 0.86$). Furthermore, the presence of RA in trace amounts was not correlated with ROL levels in both ROL-treated and untreated tissues (average $r = -0.1$, $p > 0.3$). Significantly, the major metabolites of RA (i.e., 4-hydroxy-RA, 4-*oxo*-RA) were not found to be increased in 0.4% ROL-treated skin **(Figs 7 and 8)**. RE was found in relatively small amounts (41 ± 27 ng/g wet weight), compared to ROL (305 ± 28 ng/g wet weight), in normal buttock skin (0 h). Retinyl linoleate, which accounted for 66% of total retinyl esters at time 0, remained as the predominant ester at 6 (54%), 24 (55%), and 96 (59%) h following ROL application. Other REs identified (oleate, palmitate, laurate, and stearate) each made up less than 10% of the total **(Fig 9)**.

DISCUSSION

Although topical RA has been prescribed for various skin ailments without clinically significant side effects, local skin irritation does significantly reduce compliance in certain individuals. Therefore, a naturally occurring retinoid with activity similar to RA but reduced irritancy would be desirable. When applied for 4 d under occlusion, RA produces erythema similar to that seen after longer-term clinical treatment (unoccluded once daily application). In this patch test, ROL was unable to induce significant erythema when tested up to 1.6%. This suggests that ROL may be less erythemogenic (i.e., irritating) than RA when applied for longer periods to human skin. The fact that many cosmetic products with ROL are widely used without significant problems is consistent with our observation of low irritation [3].

Despite being minimally erythemogenic, ROL induced epider-

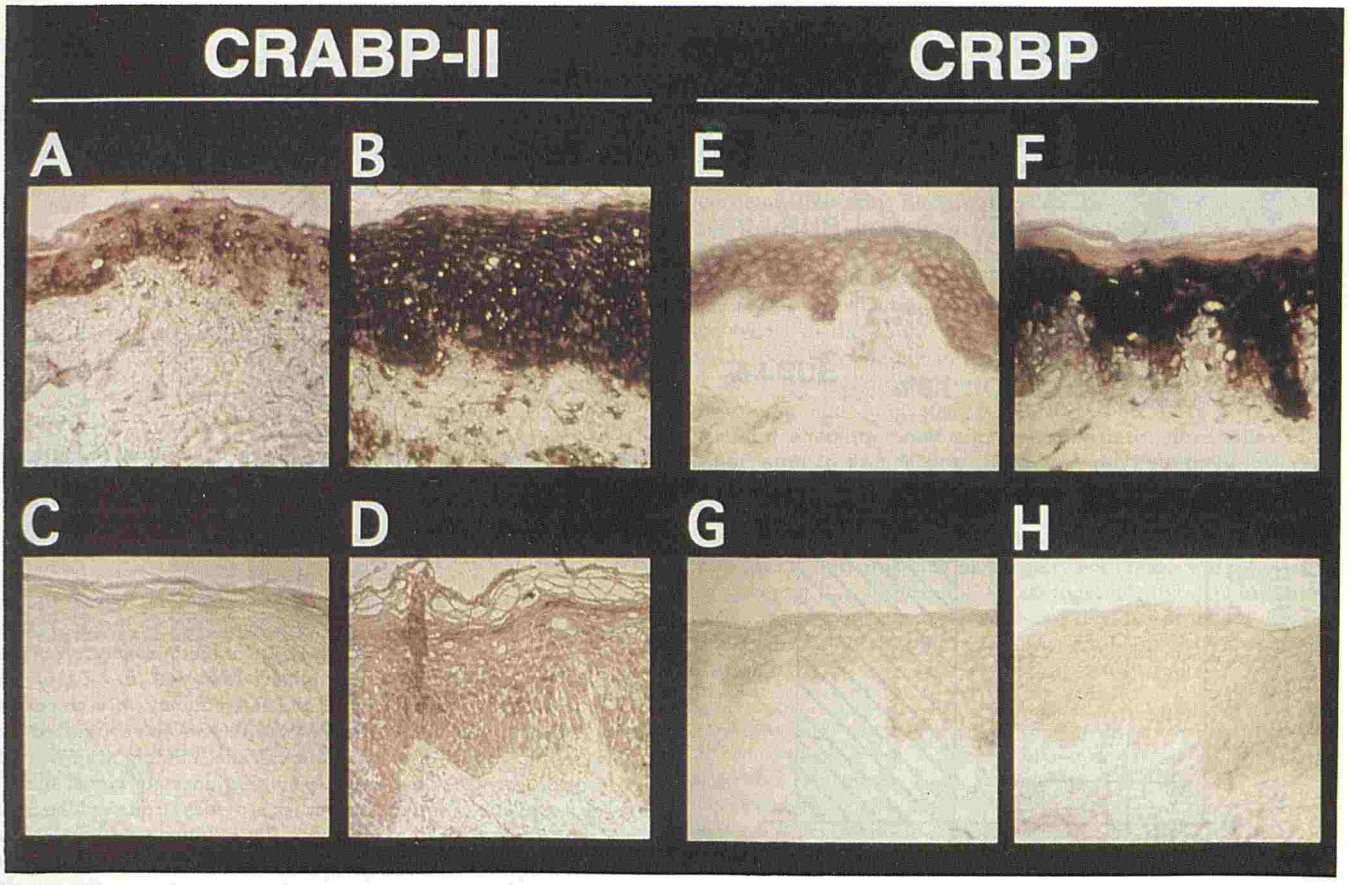

Figure 4. CRABP-II and CRBP riboprobe *in situ* hybridization. *Left*, localization of CRABP-II transcripts in human skin treated with vehicle (*A*) or ROL 1.6% (*B*). Controls are ROL-treated skin pre-incubated with ribonuclease A and probed with antisense CRABP-II riboprobe (*C*) and ROL-treated skin hybridized with a sense CRABP-II probe (*D*). *Right*, CRBP mRNA localization in human skin treated with vehicle (*E*) or ROL 1.6% (*F*). As a control, CRBP sense probe was used in vehicle (*G*) and ROL (*H*)–treated skin.

mal hyperplasia and spongiosis that were not significantly different from RA. The ability of ROL to increase epidermal thickness has been described in mouse skin [22]. However, for a retinoid to alter epidermal histology in humans without an erythematous reaction is a novel observation. Hyperplastic epidermis is a well-established histologic correlate of skin that has been irritated by certain retinoids and nonretinoid substances [13,23]. In fact, these indistinguishable clinical and histologic responses induced by RA and skin irritants such as sodium lauryl sulfate have been the basis for some to argue that non-specific irritation and not RA-specific action is responsible for clinical improvement, especially of photoaged skin, seen with topical RA. Therefore, the ability of ROL to produce epidermal morphology similar to RA (hyperplasia, spongiosis) without producing erythema is compatible with the notion that retinoid effects may be separable from irritation. Consistent with prior work, increased epidermal thickness appears to be caused by increased mitotic activity [5,11].

Human CRABP-II, like its murine counterpart [24], is expressed at the mRNA level in adult skin [17] and is induced by RA treatment [17,20]. Consistent and selective induction of CRABP-II mRNA by RA and other retinoids (and not by irritants) has made it a reliable marker for cutaneous retinoid activity *in vivo*. Furthermore, recent evidence suggests that CRABP-II gene expression may be a more sensitive indicator of retinoid biologic activity in human skin than is erythema or alterations in epidermal histology. A synthetic retinoid analog, adapalene (6-[3-(1-adamantyl)-4-methoxyphenyl]-2-naphthoic acid, CD 271) demonstrated to be efficacious in acne [25] did not induce erythema or epidermal hyperplasia but induced CRABP-II mRNA in the 4-d patch test [26]. Therefore, ROL induction of CRABP-II mRNA in a 4 day patch test may be a manifestation of retinoid biologic activity. Exact function of CRABP-II protein, whose level was also increased by ROL or RA treatment, remains enigmatic. Our data demonstrating the selective rise in CRABP-II, but not in CRABP-I protein levels by ROL and RA is in agreement with previous reports [27,28]. The proportion of CRABP-I to CRABP-II proteins (10:90) observed in the vehicle-treated skin of our subjects (n = 7) differs from the 40:60 ratio previously described in normal human skin (n = 3) [29]. Although treatment effects of the vehicle (ethanol/propylene glycol) may be partly responsible, this discrepancy in ratio may reflect a degree of variability in the expression of these proteins in normal human skin.

Induction of CRBP gene expression following ROL application suggests participation of this cytosolic binding protein in the handling of excessive ROL. Conversion of ROL to RA by successive oxidations as well as esterification of ROL to RE are believed to be mediated by CRBP, which allows interaction of bound ROL with the appropriate enzyme systems [30]. Although the ratio of apo- to holo-CRBP appears to be one important factor in determining the balance between ROL oxidation and esterification, much is still unknown about these regulatory controls. Certainly, excessive ROL delivered percutaneously would quickly saturate CRBP present in normal skin. Based on studies with liver and intestinal extracts, such a holo-CRBP dominant state would favor ROL esterification while inhibiting enzymes involved in the de-esterification process [31]. The time-dependent rise in RE content we observed following ROL application is apparently similar to the regulatory role of holo-CRBP in liver. However, it is

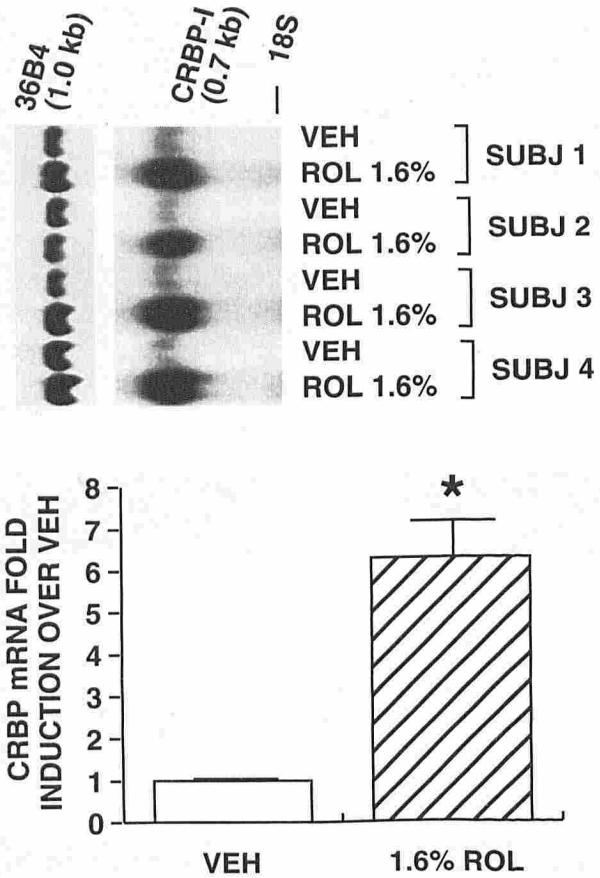

Figure 5. Northern analysis of CRBP-I gene expression following 4-d occlusive ROL treatment. Results shown are from four volunteers. Normalized CRBP-I mRNA levels were determined as described in *Materials and Methods*. *p < 0.01 versus vehicle.

not yet clear whether the esterification process in human skin is mediated by lecithin:retinol acyltransferase, which can utilize ROL bound to CRBP, or by acyl-CoA:retinol acyltransferase, which does not utilize holo-CRBP [31].

Although ROL was capable of inducing RA-like responses (epidermal thickening, enhanced expression of CRABP-II and CRBP genes), a much higher concentration of ROL than RA was

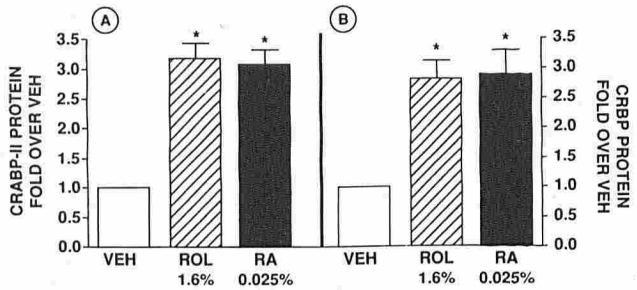

Figure 6. ROL or RA treatment of human skin *in vivo* causes increase in CRABP-II (*A*) and CRBP (*B*) levels as measured by ligand binding to CRABP protein resolved by fast protein anion exchange liquid chromatography (*A*) and Western analysis (*B*), respectively. Data represent mean ± SEM. n = 7 for CRABP-II and n = 6 for CRBP protein measurements. VEH, vehicle. *p < 0.005 versus vehicle. Assay conditions and analytical procedures used are described in *Materials and Methods*.

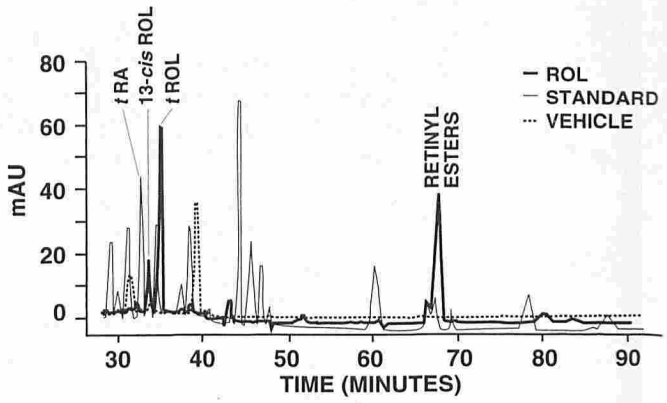

Figure 7. Reverse-phase HPLC separation of retinoids as described in *Materials and Methods*. Representative chromatograms from ROL- and vehicle-treated skin are superimposed and retention times for retinoid standards are indicated.

needed to achieve similar results. For example, based on the concentrations of ROL (0.01–0.05%) and RA (0.001%) [11] at which half-maximal CRABP-II mRNA induction were observed, ROL is about twentyfold less potent than RA in this *in vivo* bioassay. The difference in ROL and RA potency with respect to these responses may reflect the need for ROL to be converted first to RA before initiating the retinoid cascade. Finding at most trace levels of RA in all ROL-treated specimens does not contradict the need for conversion to RA, but rather provides strong evidence for tight regulation of ROL oxidation to RA in human epidermis. This two-step process is also believed to be mediated by CRBP [30]. However, it is clear that in ROL-sufficient epidermis, the esterification pathway predominates. After ROL application, the lack of detectable increases in 4-hydroxy-RA or 4-*oxo*-RA, the two major metabolites of RA, suggests that the absence of increased RA content is due to restricted RA synthesis rather than from greater RA formation compensated by proportionately increased RA breakdown. Although not detected in the work presented here, we have recently demonstrated that ROL (1.6%) treatment of normal skin *in vivo* for 4 d will induce a fourteenfold increase in RA 4-hydroxylase activity, which is specific for the all-*trans* isomer of RA (Duell, Kang, Voorhees, to be published). Therefore, by both

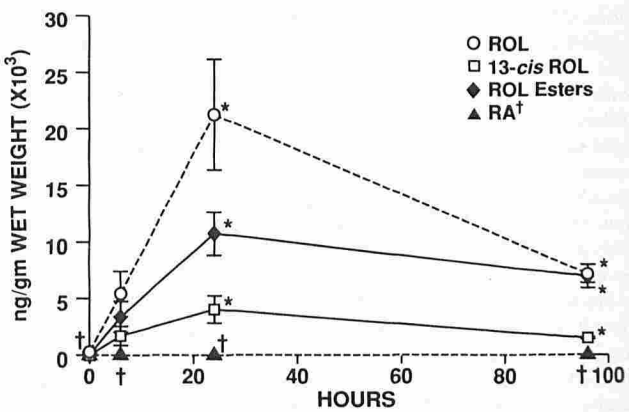

Figure 8. Time course of epidermal retinoids in human skin following ROL (0.4%) treatment. Data represent mean ± SEM levels of ROL, 13-*cis* ROL, retinyl esters, and RA measured after 0, 6, 24 and 96 h of 0.4% ROL application (n = 5). †Detection of RA in only trace amounts in one or two of five specimens at each time point. In the remainder of the specimens, RA was undetectable (detection limit, 1 ng). *p < 0.01 versus baseline.

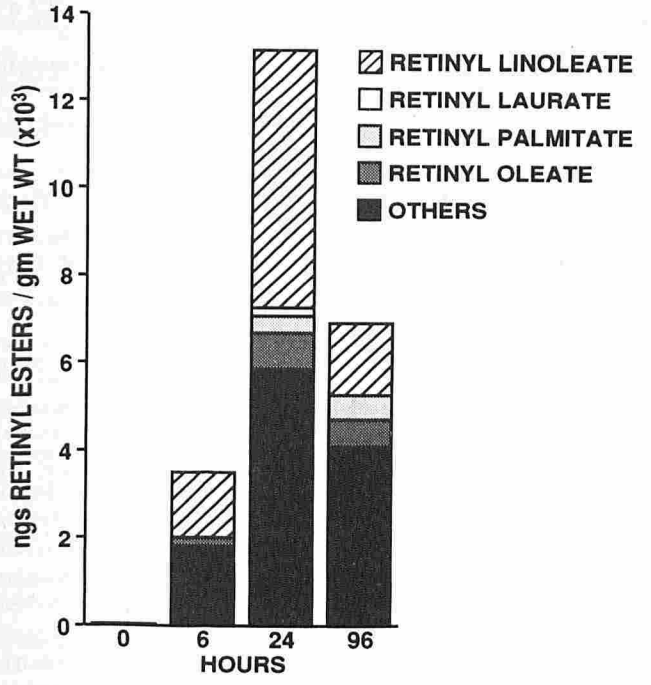

Figure 9. Composition of retinyl esters in stripped human skin epidermis *in vivo* prior to and 6, 24, and 96 h following 0.4% ROL treatment (n = 5).

restricting RA synthesis as well as inducing the enzymatic system that metabolizes RA, exquisitely fine control of RA levels (below the detection limit of our HPLC system, 1 ng) is achieved following ROL treatment of human skin.

We do not provide direct evidence for the conversion of ROL to RA in this study. However, activation of two retinoic acid responsive genes (CRABP-II and CRBP) by topical ROL strongly supports this idea. The human CRABP-II gene contains a retinoic acid responsive element in its far upstream region [32]. Although not yet characterized in humans, a retinoic acid responsive element similar to that described in the mouse CRBP gene [33] may exist within the human gene. Therefore, CRABP-II (and perhaps CRBP) mRNA induction by ROL indicates *in vivo* conversion of ROL to RA, which by binding to nuclear retinoic acid receptors/retinoid X receptors can directly induce CRABP-II (and likely CRBP) gene expression. Although ROL has been shown to bind to RARs *in vitro* [34], evidence indicates that it is unable to directly activate retinoic acid receptor–mediated gene transcription [35–37]. However, the possibility that as yet unidentified metabolite(s) of ROL, acting as ligand for RAR and/or RXR, may participate in induction of the CRABP-II gene cannot be excluded.

In vitro studies with cultured human keratinocytes provide more direct evidence for conversion of ROL to RA. As in our *in vivo* studies, cells provided with exogenous [^{3}H] ROL synthesize only small amounts (less than 1% of cell-associated radioactivity) of RA. In keratinocytes, ROL activates an RAR-dependent reporter gene ($RARE_3$-*tk*-CAT). This reporter activation and synthesis of RA are markedly inhibited by citral, an inhibitor of ROL metabolism to RA. These *in vitro* data suggest that in human keratinocytes, ROL requires conversion to RA to trigger RA target genes [38]. Similar approach using inhibitors of ROL oxidation may demonstrate more directly the necessary conversion of ROL to RA to deliver retinoid activity *in vivo*.

If indeed the RA-like effects of ROL *in vivo* are mediated by RA formed from ROL (as is the case in keratinocytes *in vitro*), the lack of a detectable increase in RA content following ROL application may be explained by tightly controlled, low-level (undetectable or trace) conversion of ROL to RA at a specific physiologically relevant site(s). This process of RA formation in the right amount at the correct locations is most likely enabled by the cellular retinoid-binding proteins. The efficiency of such a mechanism is remarkable especially when one compares it to the situation following RA treatment. For RA, following 4 d of occlusive treatment, microgram quantities of RA are recovered in stratum corneum-free skin. Because the detection limit of our HPLC is 1 ng, the RA content of epidermis is approximately a thousandfold less in ROL- versus RA-treated skin. Thus, the systemic absorption of ROL-derived RA would be markedly less. Although some ROL may be absorbed after topical ROL application, the quantity would be trivial in the context of diet-derived microgram quantities of ROL normally present in blood [39].

ROL application, without causing significant erythema, produces histologic and molecular (CRABP-II and CRBP mRNA and protein) alterations that are very similar to those following RA application to human skin. However, the extremely low (in fact unmeasurable to trace) RA levels following ROL application, in comparison to microgram quantities of epidermal RA (i.e., approximately a thousandfold more RA) following direct RA application, provides a substantial margin of safety with respect to the possibility of systemic RA absorption. Taken together, the data indicate that ROL appears to be a prohormone of RA that produces classic RA effects in skin without significant erythema or other signs of irritation.

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